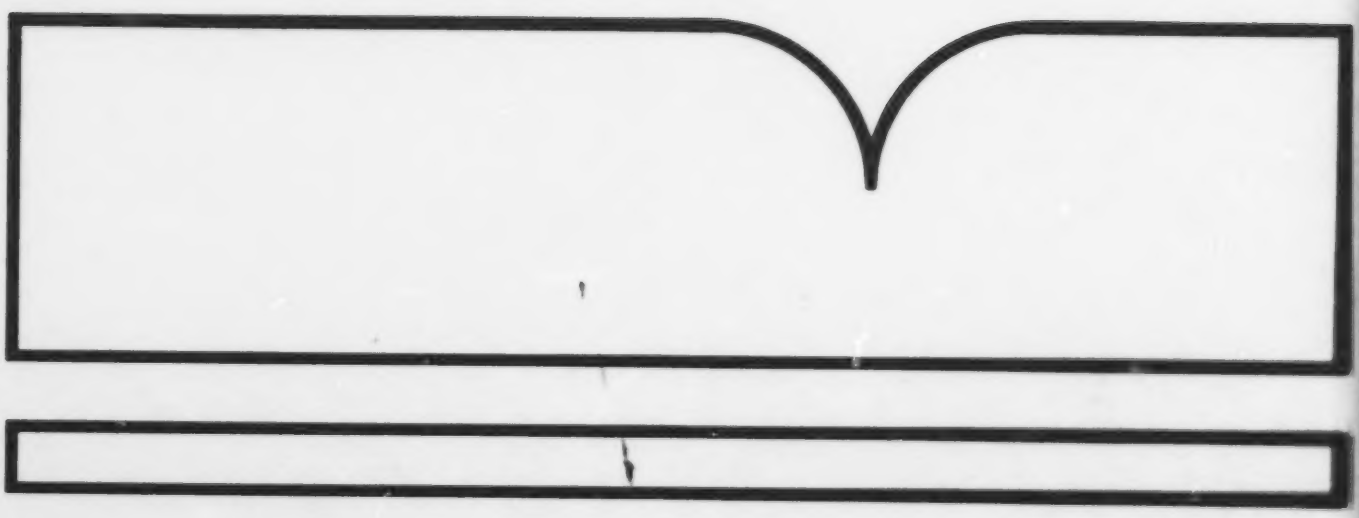
PB81-233801

Experimental Infection of the Respiratory Tract with Mycoplasma Pneumoniae

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

Jul 81



U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/J-81-028	2. JOURNAL ARTICLE	3. RECI PB81-233801
4. TITLE AND SUBTITLE: Experimental Infection of the Respiratory Tract with Mycoplasma pneumoniae		5. REPORT DATE July 1 81 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): Ping-Chuan Hu, Jerome M. Kirtz, Donald E. Gardner, and Dwight A. Powell		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Bat 7.		10. PROGRAM ELEMENT NO. A9GA1A 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS: Health Effects Research Laboratory U.S. Environmental Protection Agency Research Triangle Park, N.C. 27711		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

Published In: Environmental Health Perspectives 35: 101-106, 1980

16. ABSTRACT

M. pneumoniae, a common human respiratory pathogen, has been studied experimentally for years using intranasal inoculation of the golden Sytrian hamster. Because of recent evidence outlining the role in pulmonary immune development of particle size and depth of mycoplasma deposition in the hamster lung, we developed an aerosol chamber for the reproducible aerosolization of radiolabeled M. pneumoniae. Organisms were labeled to high specific activity by the incorporation of ^{3}H-oleic acid and aerosolized under airflow and humidity conditions creating a mean particle diameter of 2.0 μm. Under these conditions, viable mycoplasmas were reproducibly and evenly distributed to all major lobes of the lung. Examination of radioactive clearance and organism viability within the lung during the first 48 hr after aerosolization have suggested a minimal role for macrophage mycoplasmacidal activity and a more prominent role for ciliary clearance. Data from aerosol infections of hamsters with radio-labeled M. pneumoniae should provide a unique opportunity to examine in a highly controlled manner the effects of air pollutants on the initial stages of infection as well as effects on the development of pulmonary immunity and histologic alterations.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT: RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO. OF PAGES
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (9-73)

EPA-J-81-028
Journal Article

Environmental Health Perspectives
Vol. 35, pp. 101-106, 1980

Experimental Infection of the Respiratory Tract with *Mycoplasma pneumoniae*

by Ping-Chuan Hu,* Jerome M. Kirtz,* Donald E. Gardner,* and Dwight A. Powell†

***M. pneumoniae*, a common human respiratory pathogen, has been studied experimentally for years using intranasal inoculation of the golden Syrian hamster. Because of recent evidence outlining the role in pulmonary immune development of particle size and depth of mycoplasma deposition in the hamster lung, we developed an aerosol chamber for the reproducible aerosolization of radiolabeled *M. pneumoniae*. Organisms were labeled to high specific activity by the incorporation of ^{3}H-oleic acid and aerosolized under airflow and humidity conditions creating a mean particle diameter of 2.0 μm. Under these conditions, viable mycoplasmas were reproducibly and evenly distributed to all major lobes of the lung. Examination of radioactive clearance and organism viability within the lung during the first 48 hr after aerosolization have suggested a minimal role for macrophage mycoplasmacidal activity and a more prominent role for ciliary clearance. Data from aerosol infections of hamsters with radio-labeled *M. pneumoniae* should provide a unique opportunity to examine in a highly controlled manner the effects of air pollutants on the initial stages of infection as well as effects on the development of pulmonary immunity and histologic alterations.**

Introduction

Mycoplasma pneumoniae is one of the most common pathogens of the human respiratory tract. Infection with this agent is the leading cause of acute pneumonitis in high contact environments such as college campuses and military bases, as well as being a common cause of respiratory illness in children (*1*). Illnesses associated with *M. pneumoniae* infections range from mild upper respiratory infection, through tracheobronchitis, to acute pneumonia (*2*). These agents have been associated with exacerbations of chronic bronchitis in adults with chronic obstructive pulmonary disease and with severe illnesses in compromised hosts such as patients with B-cell immunodeficiency (*3*) or sickle cell anemia (*4*). Because of the prevalence and seriousness of *M. pneumoniae* associated respiratory illness, it is important to determine the effects of air pollution on the interaction between this organism and host respiratory tissues. Fortunately, the Golden Syrian hamster provides an established animal model for *M. pneumoniae* infection (*5*). The purpose of this paper is to review briefly the features which have made this animal model so ideal for studying *M. pneumoniae* pathogenesis, to describe a recently developed method for studying this model using aerosolized, radioactively labeled *M. pneumoniae*, and finally, to describe some observations on the deposition and clearance of aerosolized mycoplasmas within hamster lungs.

Review of Animal Model *Mycoplasma pneumoniae* Infection

Studies on the pathogenesis of *M. pneumoniae* infection in the Golden Syrian hamster have been conducted both *in vitro* and *in vivo*. One main thrust of the *in vitro* work has involved the study of isolated tracheal rings infected with *M. pneumoniae* (*6, 7*). Through these studies it has been established that *M. pneumoniae* is a filamentous organism, lacking a cell wall, but possessing a unique tip structure responsible for its attachment to its primary host target tissue, ciliated respiratory epithelium (*6*). This

*Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711.
†Department of Pediatrics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514.

attachment causes a variety of adverse effects on the ciliated cell including alterations in cellular metabolism (*8, 9*), changes in cellular ultrastructural morphology (*6*), and eventually, disorganization of ciliary activity and loss of cilia from the epithelial surface (*10*).

Additionally, examination of the *in vitro* interaction between *M. pneumoniae* and isolated alveolar macrophages (*11, 12*) has shown that *M. pneumoniae*, like several other mycoplasma species (*13*), has the capacity to resist phagocytosis unless opsonized with specific antibody or complement. This feature exists despite the absence of an identifiable capsule or cell wall, the key components essential for bacterial antiphagocytic capacity.

Studies of the effects of *M. pneumoniae* infection in the Golden Syrian hamster began with the work of Dajani et al. (*5*). Following intranasal inoculation of 0.2 ml of virulent organisms, the hamster develops a bronchopneumonia which is very similar to human disease. At the peak of infection, the histologic picture consists of scattered areas of peribronchial and to a lesser extent perivascular round cell infiltrate. The intraluminal exudate which develops is composed of mononuclear and polymorphonuclear phagocytes (*14*). Pulmonary disease is evident by day 3 to 7 post infection, peaks on the day 10 to 14, and begins to resolve by the third week of infection. All animals ultimately recover from the primary infection with a return to normal of the lung histology and complete clearance of the organisms. The development of IgG antibodies, as measured by complement fixation and growth inhibition, follow a time course similar to human disease. When animals have recovered from a primary infection, they are left with a long-lasting relative immunity against reinfection. While the precise mediators of this immunity remain undefined, it is known that stimulation of local pulmonary immune mechanisms is more important than stimulation of systemic immunity (*15*).

This concept was substantiated following the recent article by Jemski et al. (*16*), outlining the role of aerosol particle size and site of deposition of *M. pneumoniae* on the development of respiratory disease and immunity in hamsters. In their report, representing the only published account of an animal model aerosol infection by *M. pneumoniae*, the authors demonstrated that the response of hamsters to *M. pneumoniae* infection was determined by the site of deposition of organisms and by the quantity of organisms introduced directly into the lower respiratory tract. The variables which were studied included *M. pneumoniae* delivered as a large particle aerosol, with a mass median diameter of 8 μm, a small particle aerosol with a mass median diameter of 2.3 μm, or intranasal inoculation of 200 μl, 20 μl, or 2 μl of organisms. Only the large volume intranasal inoculum and the small particle aerosol resulted in deposition of mycoplasmas into the lungs. The other three routs of infection colonized only the upper airways. The most significant disease as measured by percent of animals with pneumonia and overall mean pneumonia score, was found in animals with the large volume intranasal inoculum. Animals with small volumes but containing the same number of mycoplasmas, administered only to the upper airway, developed little or no pneumonia. This observation suggested to these authors that the development of disease in the hamster may depend largely on the initial number of organisms delivered directly to the lower respiratory tract. Thus, it would seem that initial deposition and rate of organism clearance may be very important determinants of disease development.

Similarly, the initial location of organism deposition within the respiratory tract was critical in the generation of a protective immune response. In this segment of the study, all animals were allowed to recover 4-8 weeks following primary infection and then challenged with a large volume inoculum of intranasal organisms. While any route of infection conferred at least some protection against disease development, maximal protective immunity was seen only in those animals given initial exposure which provided inoculation of the lower respiratory tract — that is either by large volume intranasal inoculation or by small particle aerosol. Thus, again, initial deposition and pulmonary clearance would seem to be crucial events in determining the generation of protective immunity.

Aerosol Chamber Development

With these data in hand, we decided that the evaluation of air pollutant effects on disease and immune response to *M. pneumoniae* infection in the hamster must be studied such that the initial deposition and clearance could reproducibly be evaluated. To accomplish this, an aerosol exposure system was developed in which viable mycoplasmas, tagged with a radioactive marker could safely and efficiently be delivered in an aerosol with a particle mass diameter that would assure deposition deep within the lower respiratory tract. One of the main criteria considered in designing the aerosol inhalation chamber was to be able to have a high concentration of radioactively labeled *M. pneumoniae* introduced into the lungs to make deposition and clearance studies possible. Thus, we designed a chamber of small volume with a low total air flow rate to maximize the organism's air concentration during a 25-min exposure period. In Figure 1 is shown a

schematic diagram of the inhalation chamber. Compressed air is filtered and passed into rotometers which control air flow rate into the nebulizer and the drying column. Air enters this column through two tangential inlets causing a turbulence which causes evaporation of water from the nebulized aerosol reducing the particle size. From the drying column, the combined air streams enter the center of the chamber and then flow into the two side exposure sections where inhalation occurs.

Since *M. pneumoniae* are human pathogens and were radioactive in many experiments, stringent safety precautions were taken to filter all exhaust air and provide adequate containment of the aerosolized organisms. This was accomplished by housing the nebulizer, mixing column and exposure chamber, inside a stainless steel Rochester chamber, and venting all exhaust air through several HEPA particle filters. Sampling of the aerosol particle size was performed with a seven-stage low flow rate cascade Lovelace impactor.

To prepare radioactive-labeled *M. pneumoniae* for aerosolization, organisms were grown on the inner surface of glass bottles, labeled with tritiated oleic acid, washed extensively to remove unincorporated radiolabel, and scraped into M199 containing 1% horse serum. During a single run, 4 ml of suspended organisms containing approximately 10^{10} colony-forming units (CFU) were aerosolized over a 25-min time period at 21°C and 27% RH. Following this, 4 ml of saline were aerosolized for an additional 25 min to complete the total exposure. To attain the desired particle size, the nebulizer flow rate and dilution air flow rate was 5.5 and 11.5 l/min, respectively.

In Table 1 is shown the high degree of particle size reproducibility accomplished with this chamber. Here, results of experiments occurring over a one-year time period are presented to demonstrate the consistency of the count median aerodynamic diameter. The latter value, reflecting the particle size reaching the hamster lung is within the size range

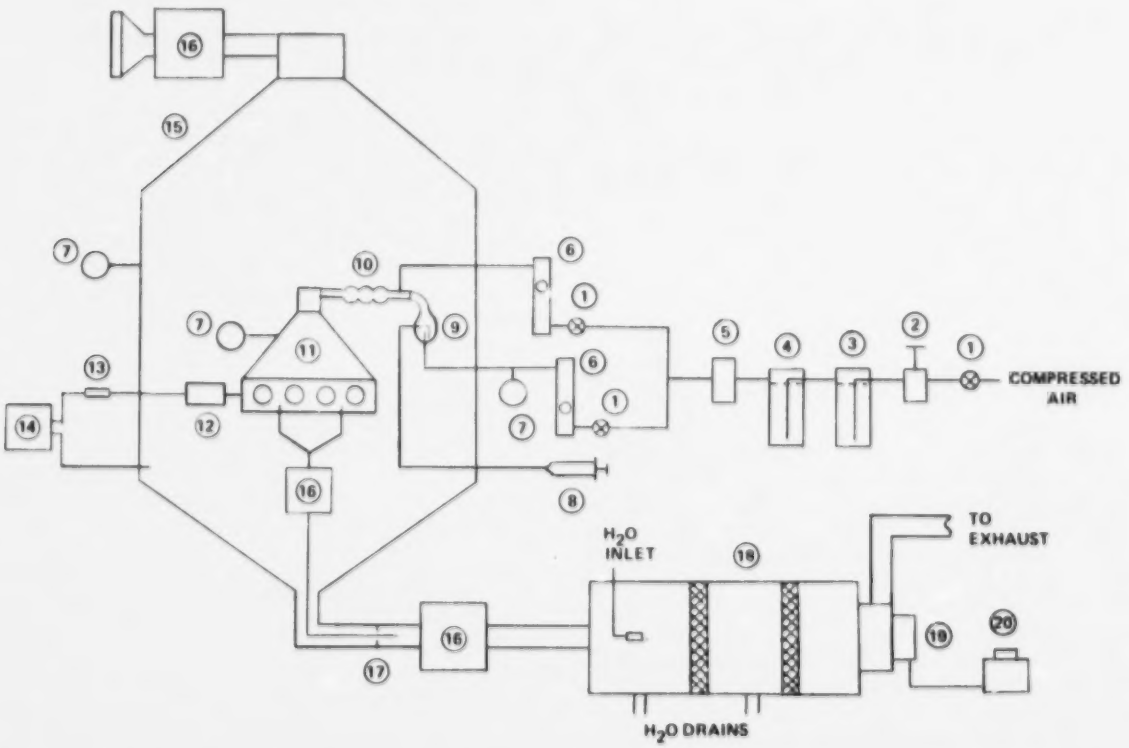


FIGURE 1. Schematic diagram of *Mycoplasma pneumoniae* aerosol inhalation chamber: (1) valve; (2) pressure regulator; (3) silica gel scrubber; (4) charcoal scrubber; (5) canister HEPA filter; (6) rotameter; (7) pressure gauge; (8) syringe; (9) nebulizer; (10) mixing column; (11) exposure chamber; (12) impactor; (13) sampling orifice; (14) sampling pump; (15) containment chamber; (16) HEPA filter; (17) orifice; (18) fume scrubber; (19) exhaust blower; (20) transformer.

Table 1. Size distribution data for *M. pneumoniae* aerosol in eight port chamber.

Exposure date	Count median aerodynamic diameter (CMAD)	Geometric standard deviation (σ_g)	Mass median aerodynamic diameter (MMAD)
08-19-77	1.8	1.5	2.9
10-11-77	1.6	1.4	2.2
10-20-77	1.7	1.4	2.5
10-25-77	1.7	1.4	2.5
12-01-78	1.7	1.5	2.7
01-17-78	1.4	1.4	2.0
01-19-78	1.4	1.5	2.1
01-21-78	1.3	1.4	1.9
08-04-78	1.5	1.4	2.2
08-07-78	1.5	1.4	2.1
08-14-78	1.6	1.4	2.2
08-16-78	1.6	1.4	2.3
Average ± SD	1.57 ± 0.15	1.42 ± 0.05	2.3 ± 0.3

Table 2. Comparison of *Mycoplasma pneumoniae* concentrations in nebulizer solution, air sample, and hamster lungs.

Exposure date	Nebulizer solution concentration, cpm/10 μl	Air sample concentration, cpm/ml air	Average hamster lung content at I_0, cpm/lung
8-19-77	2.5×10^5	15.6	6,241
8-22-77	0.85×10^5	4.4	2,358
8-31-77	2.4×10^5	13.6	6,097
9-08-77	2.8×10^5	32.1	13,135
10-11-77	1.2×10^5	8.9	4,453
10-13-77	1.2×10^5	7.9	4,210
10-20-77	1.2×10^5	—	2,403
10-25-77	2.2×10^5	13.4	6,138
12-01-77	0.94×10^5	—	6,807
01-17-78	1.2×10^5	—	5,424
01-19-78	1.0×10^5	12.6	5,661
01-21-78	0.78×10^5	6.9	4,815
08-04-78	3.6×10^5	41.5	15,937
08-07-78	4.5×10^5	39.6	20,283
08-14-78	3.0×10^5	26.7	15,581
08-16-78	3.2×10^5	—	22,010

which would assure deposition within the distal airways of the lower respiratory tract.

The correlation between the radioactive concentration in the nebulizer solution and the radioactive counts measured within the central aerosol chamber and found in the hamsters' lungs at the end of aerosol exposure is shown in Table 2. As shown by the regression analyses in Table 3, there was greater than 90% correlation between the concentration of mycoplasmas within the chamber air and that found in hamster lung.

Of potentially more importance, however, was the similarity of deposition for all animals during each aerosol exposure. If any ports were found to provide variable exposure or exposure consistently above or below the mean, the value of this exposure chamber for comparative studies would be questionable. Figure 2 demonstrates graphically the excellent correlation of exposure in all eight animals studied in each of four successive experiments. These data are tabulated in Table 4 where it can be seen that the coefficient of variation of the average for the eight animals in any one experiment varied from 0.14 to 0.19 cpm/mg of dry lung weight.

One important question in an aerosol system such as this is the extent to which aerosolized organisms remain viable. To determine how many organisms were killed by the aerosolization process, the percent of mycoplasmas found to be viable upon deposition in the hamster lung immediately at the end of aerosol exposure was multiplied by the specific activity (CFU/cpm) of the initial aerosol suspension to determine the theoretical number of viable organisms delivered to the respiratory tract. The CFU of organisms recovered divided by this theoretical number revealed the approximate actual survival rate which averaged 55%.

Deposition and Clearance of *Mycoplasma pneumoniae* in the Hamster Lung

Following the achievement of technical reproducibility in the mycoplasma aerosol chamber, clearance studies were undertaken in nonimmune hamsters. In these studies, pulmonary clearance, as measured by the removal of radioactively tagged mycoplasmas from the lung was compared with pulmonary mycoplasmacidal capacity, as measured by the rate of decline of mycoplasma viability within the lung. One aim in these studies was to compare the clearance and viability curves with similar data published on animal models infected with aerosolized bacteria (*17, 18*). Prior studies have examined *in vivo* clearance of radioactively labeled bacteria administered to animal lungs by aerosol exposures which provide particles of approximately 2 μm mass median diameter. Under these circumstances, deposition occurs primarily at the alveolar level and pro-

Table 3. Regression analysis of data in Table 2: $Y = A + BX$, r = correlation coefficient.

X vs. Y	A	B	r^2
Nebulizer solution vs. lung content	−995	0.0483	0.76
Nebulizer solution vs. air concentration	−3.08	1.00×10^{-4}	0.84
Air concentration vs. lung content	719	431	0.92

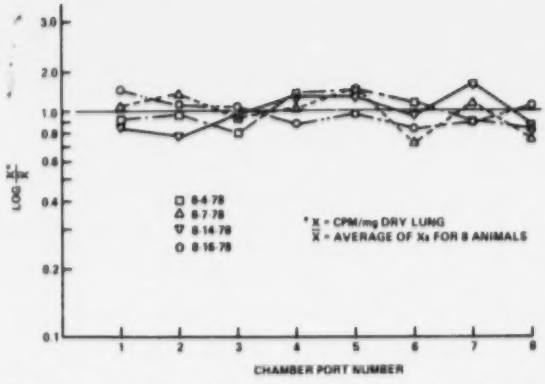


FIGURE 2. Chamber position vs. normalized deposition of radioactive-labeled *Mycoplasma pneumoniae*.

vides the capacity to analyze simultaneously physical removal and viability of the intra-alveolar organisms.

With most bacteria, the rate of transport or physical clearance of radioisotope out of the lung occurs more slowly than the decline in the number of viable bacteria. This has led to the theory that physical removal of bacteria plays a relatively minor role in the early defense of the lung and that a more important pulmonary defense mechanism involves alveolar macrophage engulfment and intracellular killing of the inhaled bacteria (*19*).

Table 4. Specific activity of radioactive-labeled *Mycoplasma pneumoniae* deposited in hamster lungs.

Exposure date	Average cpm for 8 animals	Average cpm/mg dry lung for 8 animals	Standard deviation of individual, cpm/mg dry lung	Coefficient of variation
8-04-78	15,937	122.5	19.8	0.16
8-07-78	20,283	166.9	32.0	0.19
8-14-78	15,581	119.0	22.6	0.19
8-16-78	22,010	162.4	22.2	0.14

Figure 3 shows the viability of *M. pneumoniae* deposited in the total lung tissue. There was little decline in the viability of *M. pneumoniae* during the first 8 hr after aerosolization. Only after this time period was there a decline in viability, such that by 24 hr, the viable micoplasmas in the lung were reduced by 90%. Thus, during the initial 8 hr, when macrophage engulfment and intracellular killing play the chief role in intrapulmonary removal of inhaled bacteria, little change in *M. pneumoniae* viability was noted. However, the number of viable organisms began to increase significantly by 48 hr following the initial infection, which indicates that the rate of replication was faster than the rate of clearance by host defense mechanisms. At 10 days of post-infection, the number of viable mycoplasmas reached a plateau of 10^7 organisms per lung, and then started to decline by 6 weeks after the infection. Histological sections taken from animals 20 days post-infection showed typical pathology including extensive peribronchial infiltration.

Figure 4 shows the clearance rate of radioactivity, which was found to be significantly more rapid than the decline in mycoplasma viability, most likely due to the rapid mucociliary clearance of nonattaching, inactivated aerosolized organisms. There was no point on the curve at which the percent of remaining radioactivity was greater than the percent of viable organisms. From these data, then, it would seem that alveolar macrophages in nonimmune hamsters play a

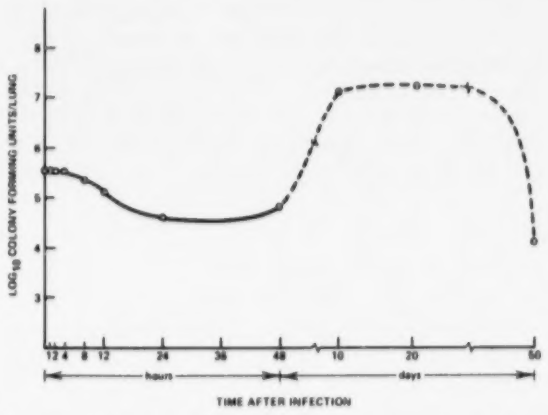


FIGURE 3. Clearance of viable *Mycoplasma pneumoniae* from hamster lungs following aerosol infection.

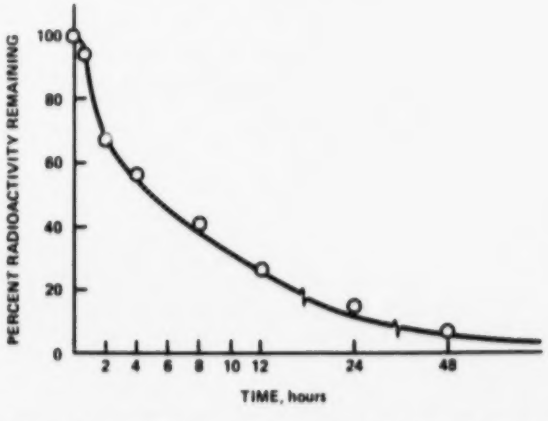


FIGURE 4. Clearance curve of radioactivity deposited as ^{3}H-oleic acid-labeled *Mycoplasma pneumoniae*.

limited role in the early pulmonary clearance of aerosolized *M. pneumoniae* and the interaction of *M. pneumoniae* and alveolar macrophages noted from *in vitro* studies are most likely representative of the relationship occurring *in vivo* (*11*). On the other hand, the role of mucociliary clearance would seem to be a more important nonspecific host defense mechanism in minimizing the initial number of viable organisms capable of establishing residence within the host lung tissues.

With the establishment of this technique for the reproducible aerosolization of viable, radiolabeled *M. pneumoniae* we are encouraged that many features of this important respiratory infection can be examined in the hamster animal model and the adverse effects of air pollution exposure determined. In addition to the ready measurement of deposition and clearance with this system, we have demonstrated that aerosol infected hamsters eventually develop histologic changes and serologic responses comparable to animals infected by 0.2 ml intranasal inoculation. Thus, air pollutant effects on the development of this form of chronic pneumonia, as well as on the generation of a protective immune response, will also be among our future research objectives.

REFERENCES

1. Denny, F. W., Clyde, W. A., Jr., and Glezen, W. P. *Mycoplasma pneumoniae* disease: clinical spectrum, pathophysiology, epidemiology, and control. J. Infect. Dis. 123: 74 (1971).
2. Clyde, W. A., Jr. Mycoplasma pneumonia. In: Medical Care of the Adolescent, J. R. Gallagher, Ed., 3rd ed., Appleton-Century-Crofts, New York, 1976.
3. Foy, H. M., Ochs, H., Davis, S. D., Kenny, G. E., and Luce, R. R. *Mycoplasma pneumoniae* infection in patients with immunodeficiency syndromes: report of four cases. J. Infect. Dis. 127: 388 (1973).
4. Shulman, S. T., Bartlett, J., Clyde, W. A., Jr., and Ayoub, E. M. The unusual severity of mycoplasmal pneumonia in children with sickle-cell disease. New Engl. J. Med. 287: 164 (1972).
5. Dajani, A. S., Clyde, W. A., Jr., and Denny, F. W. Experimental infection with *Mycoplasma pneumoniae* (Eaton's agent). J. Exptl. Med. 121: 1071 (1965).
6. Collier, A. M., Clyde, W. A., Jr., and Denny, F. W. Biologic effects of *Mycoplasma pneumoniae* and other mycoplasmas from man on hamster tracheal organ culture. Proc. Soc. Exptl. Biol. Med. 132: 1153 (1969).
7. Collier, A. M., Clyde, W. A., Jr., and Denny, F. W. *Mycoplasma pneumoniae* in hamster tracheal organ culture: Immunofluorescence and electron microscopic studies. Proc. Soc. Exptl. Biol. Med. 136: 569 (1971).
8. Hu, P. C., Collier, A. M., and Baseman, J. B. Alterations in the metabolism of hamster tracheas in organ culture after infection by virulent *Mycoplasma pneumoniae*. Infect. Immunol. 11: 704 (1975).
9. Hu, P. C., Collier, A. M., and Baseman, J. B. Further studies on the interaction of virulent *Mycoplasma pneumoniae* with hamster tracheal organ culture. Infect. Immunol. 14: 217, (1976).
10. Muse, K. E., Powell, D. A., and Collier, A. M. *Mycoplasma pneumoniae* in hamster tracheal organ culture studied by scanning electron microscopy. Infect. Immunol. 13: 229 (1976).
11. Powell, D. A., and Clyde, W. A., Jr. Opsonin-reversible resistance of *Mycoplasma pneumoniae* to *in vitro* phagocytosis by alveolar macrophages. Infect. Immunol. 11: 540 (1975).
12. Powell, D. A., and Muse, K. E. Scanning electron microscopy of guinea pig alveolar macrophages: *in vitro* phagocytosis of *Mycoplasma pneumoniae*. Lab. Invest. 37: 535 (1977).
13. Jones, T. C., Minick, C. R., and Yang, L. Attachment and ingestion of *Mycoplasma pulmonis* by mouse peritoneal macrophages — scanning electron microscopic observations. Am. J. Pathol. 87: 347 (1977).
14. Clyde, W. A., Jr. Models of *Mycoplasma pneumoniae* infection. J. Infect. Dis. 127: 569 (1973).
15. Fernald, G. W., and Clyde, W. A., Jr.: Pulmonary immune mechanisms in *Mycoplasma pneumoniae* disease. In: The Immunologic Reactions of the Lung, C. K. Kirkpatrick and H. R. Y. Reynolds, Eds., Marcel Dekker, New York, 1976.
16. Jemski, J. V., Hetsko, C. M., Helms, C. M., Grizzard, M. B., Walker, J. S., and Chanock, R. M. Immunoprophylaxis of experimental *Mycoplasma pneumoniae* disease: effect of aerosol particle size and site of deposition of *M. pneumoniae* on the pattern of respiratory infection, disease, and immunity in hamsters. Infect. Immunol. 16: 93 (1977).
17. Green, G. M. Integrated defense mechanisms in models of chronic pulmonary disease. Arch. Int. Med. Symp. 6: 500 (1970).
18. Rylander, R. Studies of lung defense to infection in inhalation toxicology. Arch. Int. Med. Symp. 6: 496 (1970).
19. Jakab, G. J., and Green, G. M. Regional defense mechanisms of the guinea pig lung. Amer. Rev. Resp. Dis. 107: 776 (1973).